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THE IDENTIFICATION OF FIBROUS PROTEINS IN FETAL RAT EPIDERMIS BY ELECTROPHORETIC AND IMMUNOLOGIC TECHNIQUES

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Two proteins have been identified in extracts of fetal rat skin which are related to the two major fibrous proteins of newborn rat stratum corneum. The relative amount of these proteins increases daily from the 16th to the 20th day (d) of gestation when judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoelectrophoresis using antibody to the purified fibrous protein. Two-dimensional analysis by SDS-polyacrylamide gel electrophoresis and immunoelectrophoresis demonstrates that these two proteins are the only cross-reactive species in the fetal skin from 16d to 19d development. Some additional lower-molecular-weight components can be detected at 20d and 21d. In double-diffusion analysis, cross-reactive proteins in 19d fetal extracts show partial identity but have fewer antigenic sites than proteins in 20d extracts. The 20d protein shows a reaction of identity with purified newborn fibrous protein.

Immunofluorescence studies on fetal skin support the presence of cross-reacting components at 16d development related to the newborn fibrous protein. Intensity of fluorescence increases at 18d and 20d in the spinous and granular cell cytoplasm and in the keratohyaline granules. The stratum corneum, first seen at 20d, is intensely fluorescent. The cellular localization and time of appearance of the cross-reactive proteins suggest that they may be associated with tonofilaments.

The process of keratinization in fetal rat epidermis occurs between the 16th and 20th days (d) of gestation. Morphologic changes during this time include the development of tonofilaments at 17d, keratohyaline granules at 18d, and the initial formation of stratum corneum at 20d [1–3]. Changes in the rate of DNA synthesis during morphogenesis have been reported [4]. However, the synthesis of specific proteins involved in keratinization has not been investigated.

In contrast, newborn rat epidermis has been used extensively as a model system for the study of biochemical changes during mammalian epidermal differentiation. Tonofilament and keratohyalin preparations have been isolated [5–8]; fibrous protein has been purified from the stratum corneum [9], and changes in protein content in the various cell strata have been reported [10,11]. However, the sequence of biochemical events of keratinization and the precise role of morphologic structures in this process are not yet known. The study of the initial occurence of specific differentiated proteins may help elucidate the biochemical changes which take place during keratinization. In a previous study the major fibrous proteins of newborn rat stratum corneum were purified and the antibody to them was prepared. In the present study, this antibody is used as a tool for the identification of fibrous proteins and their precursors in fetal rat skin. Investigations on the development of keratin and related proteins in chick embryo scales and feathers have been reported recently [12,13].

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MATERIALS AND METHODS

Animals. Fetal rats from Sprague-Dawley time-mated females were delivered by cesarian section. The first day after conception was considered day 0. One skin from each litter was fixed in neutral buffered formalin, embedded, sectioned, and stained with hematoxylin and eosin for age verification.

Separation of epidermis. The dorsal skin was removed and placed in Earle's balanced salt solution (EBSS). The epidermis from 20d and 21d fetuses was separated from the dermis after incubation in 0.10% trypsin at 4°C for 16 hr. A sample of 20d dermis was also obtained. Epidermal-dermal separation was not attempted on 15d to 19d skin samples.

Protein extraction. The skin samples were minced and stirred at room temperature for 1 to 2 hr in 8 M urea containing 0.1 M Tris–HCl, pH 8.5, 0.1 M 2-mercaptoethanol (2-ME), and 1 mM dithiothreitol (DTT) (UTME),

and 10 μg/ml phenylmethylsulfonylfluoride (PMSF), a protease inhibitor. Samples were homogenized, and 17d to 21d samples were reextracted for an additional 2 to 6 hr depending on age and difficulty of extraction.

Protein concentration was assayed by the procedure of Bramhall et al [14].

Carboxymethylation. Samples for immunoelectrophoretic procedures were chemically reduced in order to maximize the solubility of the fibrous proteins. Dialyzed samples were incubated for 90 min in the dark at room temperature with .025 M iodoacetic acid and 10 μg/ml PMSF. The reaction was stopped by the addition of excess 2-ME. Excess reagents were removed by dialysis against 4 M urea with 0.1 M Tris–HCl, pH 8.5, and 1 mM DTT.

SDS-polyacrylamide gel electrophoresis. The procedure of Dunker and Ruekert [15] was followed as previously described [11]. Samples for electrophoresis containing 2% SDS, 2% 2-ME, with at least 4 M urea were incubated at 100°C for 1 min. In general, 50 μg protein in 20 to 40 μl was applied to each gel. Electrophoresis was carried out at 9 mAmp per tube for 3½ hr. Gels were stained in Coomassie brilliant blue and destained as previously described [11].

Preparation of antiserum. Purified newborn rat stratum corneum fibrous protein (SCM-light chain) was used as an antigen [9]. Antiserum was prepared in a goat by repeated injection of this preparation in Freund's complete adjuvant (50 μg/ml).

Immunoelectrophoresis. The Laurell rocket method for immunoelectrophoresis was used [16]. Glass plates were coated with 0.5% agarose containing 0.05 M veronal buffer, pH 8.6. Two-thirds of this layer was removed and replaced with the agarose-veronal mixture containing antisera. Samples of known protein were applied in wells in the original agarose. Electrophoresis was carried out at 3.8 volts/cm for 18 hr. Plates were washed 24 hr in phosphate-buffered saline, 48 hr in distilled H_2O, then dried at 37°C, stained for 30 min in 0.5% Coomassie brilliant blue in acetic acid:ethanol:H_2O (10:10:80), and destained in acetic acid:ethanol:H_2O (20:10:70).

Immunoelectrophoresis of SDS-polyacrylamide gels was carried out as described above on gels sliced lengthwise and washed in distilled H_2O for 45 min at 37°C to remove excess SDS. The remaining half gel was stained in Coomassie brilliant blue and placed on the stained immunoelectrophoresis plate for photography.

Immunofluorescence. Antibody purified by affinity chromatography against the light chain of the fibrous protein of newborn rat skin stratum corneum was labeled with fluorescein isothiocyanate as described previously [9]. Direct immunofluorescence on cryostat-sectioned skin was performed on duplicate samples. As a control, one set was blocked with 3 changes of nonfluorescent antiserum; the other was treated with 3 changes of normal goat serum. Both sets were washed in phosphate-buffered saline and then treated with 3 changes of the fluorescent-labeled antibody [9]. Observations were made on a Zeiss model 1106 fluorescence microscope.

RESULTS

SDS gel electrophoresis. Protein bands with electrophoretic mobility on SDS-polyacrylamide gels equivalent to the partially purified fibrous proteins from newborn stratum corneum can be identified in the gels of fetal skin at 17d development and older. The position of these bands is indicated in Figure 1. The intensity of these bands increases with age. A third band with mobility intermediate between the two specified bands also increases in intensity from 15d to 20d development. Another prominent band with slightly greater mobility than the fibrous bands is seen at 20d. This band has been previously identified in the newborn stratum corneum and in a potassium phosphate-extracted keratohyalin preparation [17]. An SDS gel of 20d dermis proteins is included in Figure 1 for comparison with the 15d to 19d samples in which separation of dermis and epidermis was not performed. Also shown is an SDS gel of molecular weight standards.

Specificity of antiserum. The antiserum was found to be specific for epidermis. No precipitation bands are seen in Ouchterlony double-diffusion analyses with similarly prepared extracts of rat dermis, kidney, or liver (Fig. 2) or with SCM–bovine serum albumin (Fig. 3). However, a very slight reaction can be detected with SCM–BSA by immunoelectrophoresis using 20 μg SCM–BSA.

Immunodiffusion and immunoelectrophoresis of fetal extracts. Ouchterlony double-diffusion analysis of some of the fetal extracts is shown in Figure 3. The SCM-derivative extract of 16d rat skin gives little or no precipitate, while those of 18d, 19d, and 20d give two precipitate bands which fuse with each other and with the newborn fibrous protein. A reaction of identity is seen between the 20d fetal extract and the newborn fibrous proteins. How-

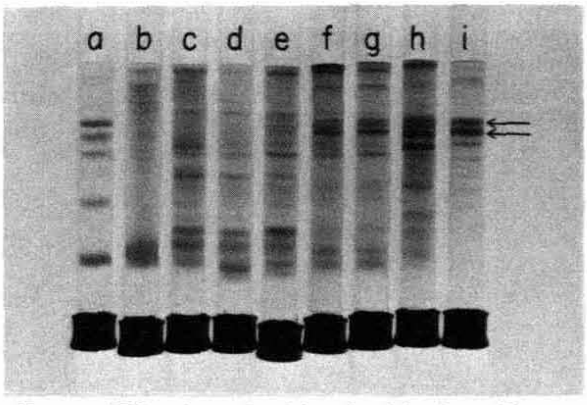

FIG. 1. SDS-polyacrylamide gels of fetal rat skin proteins and related samples. *a*, molecular weight standards; bovine serum albumin, bovine gamma globulin heavy chain, ovalbumin, bovine gamma globulin light chain, cytochrome c; *b*, 20d dermis; *c*, 15d skin; *d*, 16d skin; *e*, 17d skin; *f*, 18d skin; *g*, 19d skin; *h*, 20d epidermis; *i*, partially purified newborn rat SCM-fibrous protein.

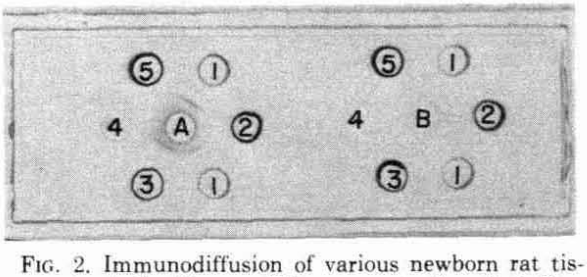

FIG. 2. Immunodiffusion of various newborn rat tissues extracted with urea–Tris–mercaptoethanol. *1*, Partially purified newborn rat SCM-fibrous protein; *2*, liver; *3*, stratum corneum; *4*, dermis; *5*, kidney. *A*, Antiserum vs newborn rat SCM-fibrous protein (light chain); *B*, normal goat serum.

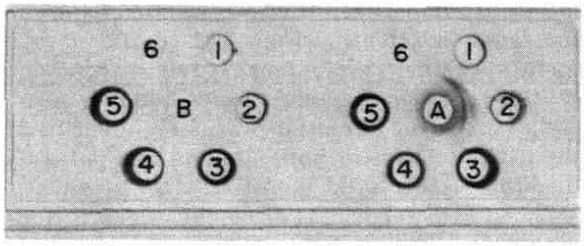

FIG. 3. Immunodiffusion of fetal extracts. *1*, Partially purified newborn rat fibrous protein (containing light and heavy chains); *2*, 20d epidermis; *3*, 19d skin; *4*, 18d skin; *5*, 16d skin; *6*, SCM-bovine serum albumin. *A*, Antiserum vs newborn rat SCM-fibrous protein (light chain); *B*, normal goat serum.

ever, strong spur formation between the 19d and 20d extracts indicates that more antigenic sites are present at 20d than at 19d.

The results of immunoelectrophoresis of fetal samples by the rocket technique are shown in Figure 4. Cross reaction with antibody to fibrous protein is detectable at 16d development and older. The amount of cross-reacting material increases with age. Formation of multiple peaks is evident in 17d to 19d samples. This suggests more than one cross-reacting protein and/or more than one state of aggregation in the UTME-solubilized samples. These possibilities are clarified by a two-dimensional technique in which proteins separated on SDS-polyacrylamide gels are subjected to immunoelectrophoresis. Such an analysis of protein from 17d, 18d, and 20d fetal skin as well as a partially purified sample of newborn rat stratum corneum fibrous protein is shown in Figure 5. UTME extracts of fetal skin from 16d to 21d specimens have been analyzed in this way. The results can be summarized as follows: the 16d extract contains one cross-reactive peak which has approximately the same mobility on the SDS gel as the light chain of the fibrous protein; the 17d, 18d, and 19d extracts contain 2 cross-reactive peaks which have the mobility on the SDS gel equivalent to the heavy and light chains of the fibrous proteins; the 20d and 21d extracts contain the same two cross-reactive proteins but show additional cross-reactive components of higher mobility (lower molecular weight) on the SDS gels. Increasing the amount of antibody 1.5- to 2-fold or the amount of extract 1.5-fold did not reveal any additional cross-reactive peaks.

Immunofluorescence. Fetal skin at 14d, 16d, 18d, 20d development and newborn rat skin were observed using direct immunofluorescence. The newborn skin (Fig. 6) shows strong fluorescence in the cornified layer and the keratohyaline granules. A less intense fluorescent reaction can be seen in the spinous layer. The 20d skin shows the same pattern of immunofluorescence as the newborn (Fig. 7*A*). The 18d fetal skin shows a positive reaction in the spinous and granular layers (Fig. 7*B*). Reaction in the 16d sample was weakly positive in the stratum intermedium. No reaction was detectable in the 14d sample.

DISCUSSION

In this study, proteins have been identified in fetal rat skin which are immunologically related to two major proteins of the newborn stratum cor-

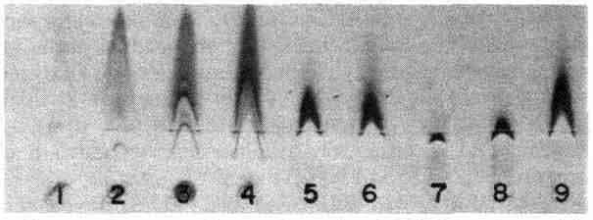

FIG. 4. Immunoelectrophoresis of SCM-fetal rat skin proteins vs antibody to SCM-fibrous protein from newborn rat stratum corneum. Wells (*1*)–(*4*) each contained 20 μg UTME extract of fetal skin; Well (*1*) 16d, (*2*) 17d, (*3*) 18d, (*4*) 19d. Wells (*5*) and (*6*) each contained 2 μg UTME extract of fetal epidermis; Well (*5*) 20d, and Well (*6*) 21d. Wells (*7*), (*8*), and (*9*) contained 0.45, 0.9, and 1.8 μg, respectively, of the partially purified newborn rat SCM-fibrous protein.

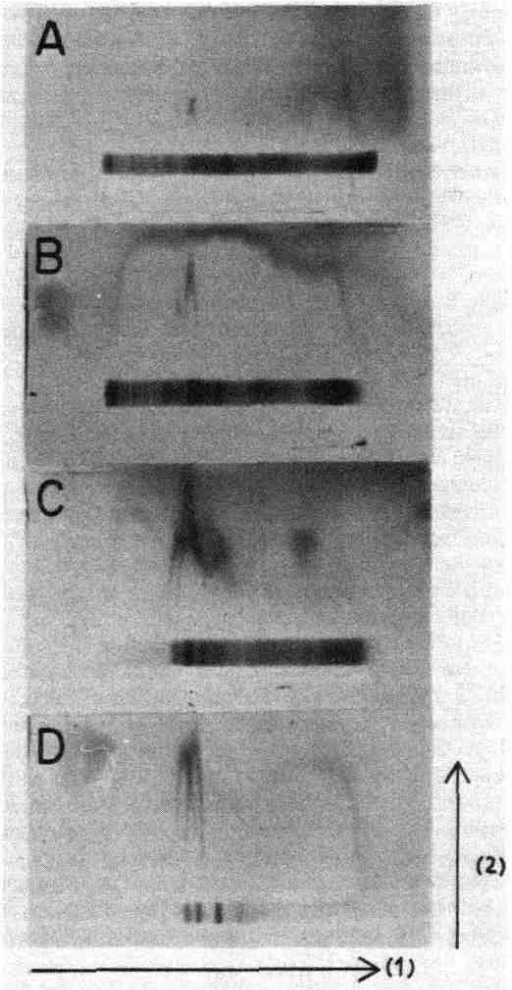

FIG. 5. Two-dimensional SDS-polyacrylamide gel immunoelectrophoresis of SCM-skin proteins. Approximately 40 μg protein was applied to an SDS-polyacrylamide gel; the gel was sliced lengthwise, washed, and one-half embedded in agarose for immunoelectrophoresis. *Arrow (1)* indicates direction of migration on the SDS gel, *arrow (2)* indicates direction of migration for immuno-electrophoresis. *A*, 17d skin UTME extract; *B*, 18d skin UTME extract; *C*, 20d epidermal UTME extract; *D*, partially purified newborn rat fibrous protein.

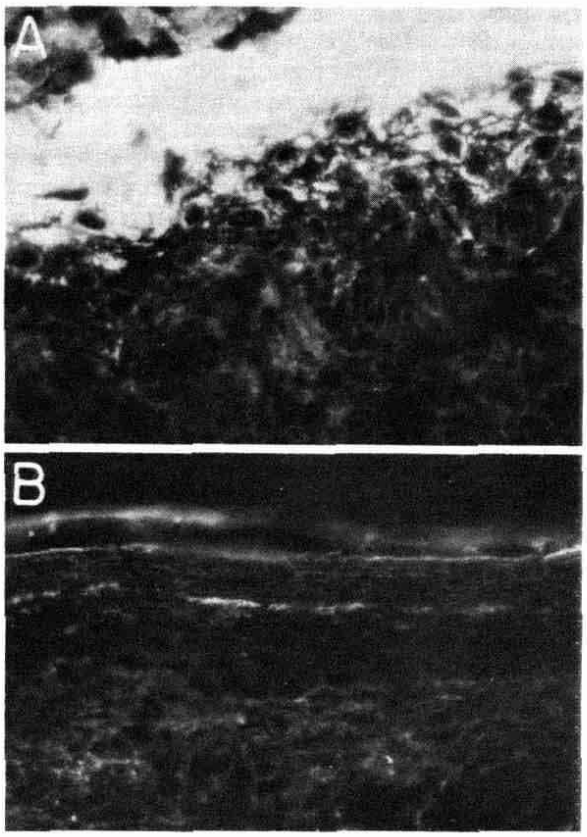

FIG. 6. Direct immunofluorescence of newborn rat skin (× 480). *A*: Section treated with normal goat serum, then with fluorescent-labeled antiserum. *B*: Blocked control: section treated with nonfluorescent antiserum then with fluorescent-labeled antiserum.

neum. Ouchterlony analysis suggests that at the time of development of stratum corneum (20d), the fetal proteins are immunologically identical to those of the newborn. However, spur formation between 19d and 20d extracts suggests a change in the cross-reactive proteins simultaneous with the formation of the stratum corneum. Two-dimensional electrophoretic–immunoelectrophoretic analysis suggests that two proteins with mobility identical to two fibrous proteins of the newborn are the cross-reactive species. At least one cross-reactive protein is present at 16d development, 4 days before the statum corneum can be identified. The relative amount of these proteins increases daily as judged by intensity of staining of bands on SDS-polyacrylamide gels, by the amount of cross-reacting material in extracts, and by immunofluorescent studies. In the newborn these proteins are the major fibrous proteins of the stratum corneum, comprising approximately 60% of the total proteins in the keratinized layer [9]. Quantitative change is not evaluated in this study because of the lack of a common unit of comparison for all samples. The dermis was not separated from the younger fetal samples; therefore, DNA content, protein content, and tissue weight are not comparable in all samples.

The technique of SDS gel electrophoresis permits the study of the monomeric form of proteins. Possible changes in native tertiary structure or changes in the state of aggregation during development will not affect the interpretation of SDS gels. The SDS gel technique by itself cannot be used to identify protein precursors of molecular weight different from the stratum corneum proteins, but when coupled with immunoelectrophoresis, precursors can be identified if they cross react with the antibody to the differentiated product.

The antibody used in these studies was elicited vs the SCM-light chain of newborn fibrous protein. It was previously shown to give a single precipitate band in Ouchterlony double diffusion with either the purified SCM-light chain or SCM-heavy chain of the fibrous protein and that these fuse to yield a reaction of identity [9]. The antibody is primarily specific for the fibrous protein, not the haptenic carboxymethyl group. This has been demonstrated by lack of cross reaction of the antibody with S-carboxymethyl-bovine serum albumin in Ouchterlony double-diffusion tests. The greater sensitivity of the rocket immunoelectrophoretic procedure revealed a barely detectable reaction with 20 μg SCM–BSA. A maximum of 20 μg of the SCM-derivative fetal protein was used for immunoelectrophoresis, thus, a false positive might be possible with this technique alone. However, essentially the same results were obtained with non-derivatized fetal proteins. In addition, identical results to those shown for the two-dimensional analysis were

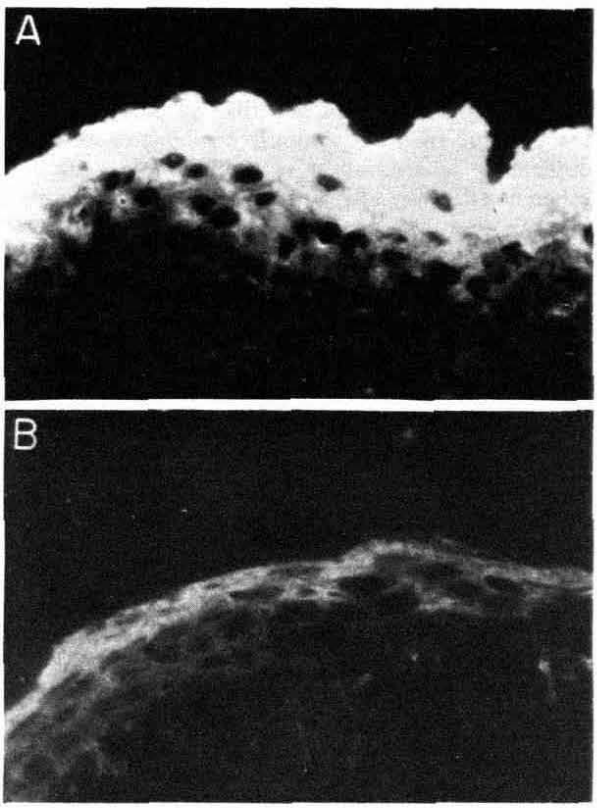

FIG. 7. Direct immunofluorescence of fetal rat skin (× 320). Sections treated with normal goat serum then with fluorescent-labeled antiserum. *A*, 20d fetal; *B*, 18d fetal.

obtained using antibody adsorbed with SCM-BSA cross-linked with ethylchloroformate.

The presence of bands on the SDS gels which have the same mobility as the fibrous proteins, and which increase with development, is suggestive evidence that these bands are the fibrous proteins. This is confirmed by two-dimensional electrophoresis–immunoelectrophoresis. Immunoelectrophoresis permits identification of one of these bands at 16d development, before it can be observed in SDS gels by protein staining.

Bands with the mobility of the fibrous proteins are the only cross-reactive proteins detected in the 16d to 19d skin. However, identity with the newborn fibrous proteins cannot be concluded. In fact, the spur formation in double-diffusion analysis between 19d and 20d extracts suggests that the protein at 19d has only antigenic sites which are identical to those at 20d, but the 20d protein has additional sites not present at 19d. This change could be due to (1) the synthesis of a new protein which coprecipitates with the previously existing protein in the double-diffusion analysis, (2) a conformation specific to stratum corneum fibrous protein, or (3) a post-translational alteration such as the binding of carbohydrate or another moiety or an alteration of an amino acid residue. The later type of change could lead to a major structural and functional change in the native aggregated state of the protein with only a negligible change in the molecular weight of the monomeric polypeptide chain. Such changes might take place during differentiation.

The 20d and 21d fetal epidermal samples contain cross-reactive species in addition to the fibrous protein heavy and light chains. These could represent precursors of the fibrous protein which are present in great enough concentration to be detectable at this stage of development. On the other hand, their lower molecular weight suggests that they might be breakdown products due to storage, enzymatic activity in the epidermis, or proteolytic activity in the crude trypsin preparation used for epidermal-dermal separation. Proteolysis by trypsin itself is unlikely because of the resistance of the fibrous proteins to the enzyme [9]. The presence of PMSF during extraction, carboxymethylation, and storage should have inhibited endogenous proteolytic activity. However, we have not eliminated the possibility of breakdown before extraction. Further studies are required to determine whether these additional cross-reactive proteins are precursors of the fibrous proteins, natural products of keratinization, or breakdown products.

The occurrence of proteins which cross-react with the newborn rat stratum corneum fibrous protein 4 days before the morphologic appearance of stratum corneum supports previous results that fibrous proteins or their precursors exist in the lower cell layers. This has been demonstrated for newborn rat [6,9,17] and for bovine snout [18–21]. The time of appearance of these proteins suggests that the morphologic component with which they are associated may be the tonofilaments. Ultrastructural studies of developing rat keratinizing epithelia suggest that tonofilaments can be identified at 17d and keratohyaline granules at 18d [3,22,23].

The results reported here are compatible with the hypothesis that tonofilaments are the components of the 16d to 18d skin containing the cross-reactive proteins. The tonofilaments become more numerous and aggregate with keratohyalin by 20d development. These changes result in an intense fluorescence in the keratohyaline granules. This intensity could be due to the biosynthesis of cross-reactive proteins, due to the concentration of cross-reactive components into a smaller area, or to some biochemical modification of keratohyalin and/or tonofilaments, or all of these possibilities. A biochemical modification could result in a change in protein conformation to that of the stratum corneum, and a concomitant exposure of additional antigenic sites resulting in the intense fluorescence of the stratum corneum.

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